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Inhibition of Werner Syndrome Helicase Activity by Benzo[*c*]phenanthrene Diol Epoxide dA Adducts in DNA Is Both Strand- and Stereoisomer-dependent*[S]

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Helicases are among the first enzymes to encounter DNA damage during DNA processing within the cell and thus are likely to be targets for the adverse effects of DNA lesions induced by environmental chemicals. Here we examined the effect of *cis*- and *trans*-opened 3,4-diol 1,2-epoxide (DE) DNA adducts of benzo[*c*]phenanthrene (BcPh) at N^6 of adenine on helicase activity. These adducts are derived from the highly tumorigenic (−)-(1*R*,2*S*,3*S*,4*R*)-DE as well as its less carcinogenic (+)-(1*S*,2*R*,3*R*,4*S*)-DE enantiomer in both of which the benzylic 4-hydroxyl group and epoxide oxygen are *trans*. The hydrocarbon portions of these adducts intercalate into DNA on the 3′ or the 5′ side of the adducted deoxyadenosine for the 1S- and 1R-adducts, respectively. These adducts inhibited the human Werner (WRN) syndrome helicase activity in a strand-specific and stereospecific manner. In the strand along which WRN translocates, *cis*-opened adducts were significantly more effective inhibitors than *trans*-opened isomers, indicating that WRN unwinding is sensitive to adduct stereochemistry. WRN helicase activity was also inhibited but to a lesser extent by *cis*-opened BcPh DE adducts in the displaced strand independent of their direction of intercalation, whereas inhibition by the *trans*-opened stereoisomers in the displaced strand depended on their orientation, such that only adducts oriented toward the advancing helicase inhibited WRN activity. A BcPh DE adduct positioned in the helicase-translocating strand did not sequester WRN, nor affect the rate of ATP hydrolysis relative to an unadducted control. Although the Bloom (BLM) syndrome helicase was also inhibited by a *cis*-opened adduct in a strand-specific manner, this helicase was not as severely affected as WRN. Because BcPh DEs form substantial amounts of deoxyadenosine adducts at dA, their adverse effects on helicases could contribute to genetic damage and cell transformation induced by these DEs. Thus, the unwinding activity of RecQ helicases is sensitive to the strand, orientation, and stereochemistry of intercalated polycyclic aromatic hydrocarbon adducts.

Helicases are enzymes that disrupt complementary strands of duplex DNA in a reaction dependent on nucleoside-5′-triphosphate hydrolysis (1–3). Evidence suggests that helicases play important roles in DNA metabolism, and a growing number of helicases have been linked to human disease (4, 5). Pathways of DNA replication, recombination, and repair are affected by DNA lesions, and the effects of helicases on such pathways are probably modulated by their interactions with chemically modified DNA. Although the mechanism for DNA unwinding has been studied for several helicases (reviewed in Refs. 1–3), very little is known regarding how specific covalently linked adducts affect helicase function.

RecQ helicases are believed to function in repairing replication forks that have been stalled by DNA damage and may also play a role in the intra-S-phase checkpoint, which delays the replication of damaged DNA, thus permitting repair to occur (6). Only limited information is available pertaining to the mechanism of DNA unwinding by RecQ helicases, and no crystallographic data are currently available. Biochemical studies of the prototype member, *Escherichia coli* RecQ helicase, indicate that the enzyme utilizes multiple interacting ATP-binding sites to unwind double-stranded DNA (7). The human *WRN* gene product, which is defective in the premature aging disorder Werner syndrome, encodes a 1,432 amino acid protein with the seven conserved motifs found in the RecQ family of Superfamily 2 DNA helicases (8). WRN[1] (9), similar to all of the other RecQ helicases characterized to date, is a 3′–5′-helicase (reviewed in Refs. 4 and 10). Thus, WRN is thought to translocate 3′–5′ along the bound single-stranded DNA residing between the duplex portions of a helicase directionality substrate. However, unidirectional translocation of WRN on a DNA lattice remains to be demonstrated. Although the DNA substrate specificity of WRN (11) and other RecQ helicases (12, 13) has been studied, the effects of DNA damage on helicases of this family have not been characterized prior to this study.

The replication (14–17) and recombination (18–20) defects observed in WRN−/− cells may reflect abnormal processing of specific structures associated with the replication fork or a DNA recombination intermediate. Our recent work demonstrated that WRN helicase is able to unwind a synthetic replication fork in the direction of the fork (11), a biochemical activity that may be important for the maintenance of fork progression. WRN was recently shown to interact functionally with DNA polymerase δ by stimulating the enzymatic rate of

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental data, Table IV, and references.

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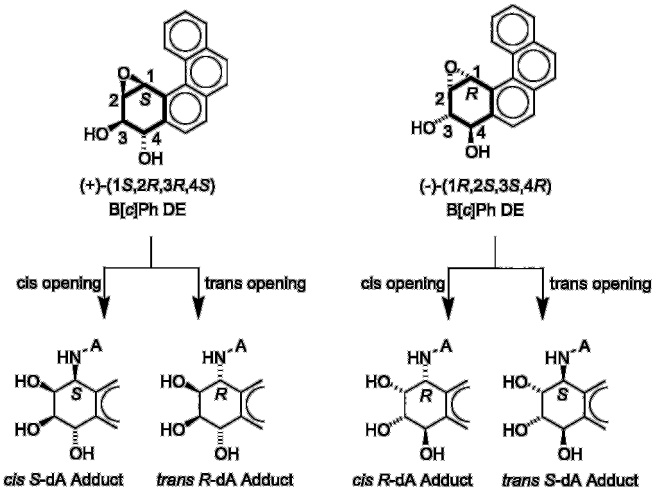
[1] The abbreviations used are: WRN, Werner Syndrome; PAH, polycyclic aromatic hydrocarbon; BcPh, benzo[*c*]phenanthrene; DE, 3,4-diol 1,2-epoxide; dA, deoxyadenosine.



SCHEME 1. **Structures of the optically active BcPh DE and their N^6-dA adducts, where *A* represents the adenine base.** The partially saturated benzo-ring of the DE that is the site of covalent attachment to the base is shown in *boldface*. Note that the absolute configuration at C-1 is retained on *cis*-opening and inverted on *trans*-opening of each DE.

nucleotide incorporation (21). It was subsequently shown that WRN facilitates polymerase δ synthesis through hairpin and tetraplex structures that impede the polymerase, suggesting that WRN may remove secondary structure at a stalled replication fork to allow polymerase δ synthesis to proceed (22). The results to be reported here suggest that WRN may not function properly to ensure replication fork progression if the fork is blocked by a covalent DNA adduct, even though WRN may disrupt other DNA structures such as tetraplexes (13, 23) or triplexes (24) that might impede a replication fork.

Polycyclic aromatic hydrocarbon (PAH) 3,4-diol 1,2-epoxide (DE)-DNA adducts have been used by us as tools to probe the interactions between other DNA-processing enzymes such as topoisomerases (25, 26) and their DNA substrates as well as to elucidate possible molecular mechanisms for the induction of cancer by these DNA lesions. In this study, we examined the effects of site-specific benzo[*c*]phenanthrene (BcPh) DE adducts at N^6 of deoxyadenosine (Scheme I) positioned centrally in the double-stranded region of a forked DNA duplex substrate on the unwinding activity catalyzed by WRN helicase. These adducts are derived by both *cis*- and *trans*-opening of the (−)-(1*R*,2*S*,3*S*,4*R*)- and (+)-(1*S*,2*R*,3*R*,4*S*)-enantiomers of the DE in which the benzylic 4-hydroxyl group and epoxide oxygen are *trans*. The DEs are examples of mutagenic and carcinogenic bay-region diol epoxide metabolites that are formed from PAH by the combined action of cytochrome P-450 and epoxide hydrolase (27, 28). Benzo[*c*]phenanthrene is a common environmental contaminant (29–33), which has been found in industrial emissions, wastewater, and food. Metabolism of the hydrocarbon on mouse skin (34) by cultured rodent embryo cells (35) or by human mammary carcinoma cells (34) led to covalent BcPh DE-DNA adducts primarily at deoxyadenosine (dA). Notably, human liver microsomes metabolize BcPh via the carcinogenic diol epoxide pathway to a greater extent than do microsomes from rat liver (36). The metabolically derived BcPh DEs react extensively with dA residues in DNA *in vitro* (37) and *in vivo* (38), and induce mutations at dA in mammalian cells (39). Such mutations provide an attractive mechanism for the induction of cell transformation leading to cancer. In particular the (−)-(1*R*,2*S*,3*S*,4*R*)-BcPh DE is one of the most tumorigenic PAH DEs identified to date (40).

A particularly useful feature of the PAH DEs is the variety of structural motifs exhibited by the different DE adducts within duplex DNA. These structural motifs have been well characterized by NMR studies. The aromatic portions of the 1*R* and 1*S* *trans*-opened BcPh DE N^6-dA adducts used in this study (see Fig. 1) intercalate between adjacent base pairs in duplex DNA (41, 42). Local stretching of the double helix accommodates the intercalated aromatic rings, and the adducted adenine retains Watson-Crick base pairing to its complement within the helix despite some buckling and propeller twisting of the adducted base pair. The non-aromatic benzo-rings are situated in the major groove, and the intercalated aromatic portion projects toward but not into the minor groove. These *trans*-opened BcPh DE-dA adducts are oriented such that the aromatic moiety of the 1*R* dA adduct intercalates on the 5′ side of the adducted adenine base, whereas the aromatic moiety of the 1*S* dA adduct intercalates on the 3′ side (41, 42). Although the solution structures of *cis*-opened BcPh DE-dA adducts in DNA have not been determined by NMR, the two *cis*-adducts in the present study are likely to exhibit the same dependence of the direction of intercalation on their configuration at C-1 as their *trans*-analogs.

The present study demonstrates that *cis*- and *trans*-opened BcPh DE-dA adducts in DNA are highly effective probes of helicase activity. The *cis-S* BcPh DE-dA adduct in the strand on which WRN is presumed to translocate was a potent inhibitor of unwinding activity, but a forked duplex containing this adduct did not sequester WRN to any greater extent than an unmodified control. The *trans-S* adduct was not as effective as the *cis-S* isomer in inhibiting translocation. Inhibition of unwinding activity is also sensitive to the orientation of the adduct (toward or away from the advancing helicase) in the displaced strand of the duplex region, which is opposite to the strand on which the helicase translocates.

MATERIALS AND METHODS

Proteins—Recombinant hexahistidine-tagged WRN protein (wild-type, WRN-E84A) was overexpressed using a baculovirus/*Sf9* insect system and purified as described previously (43). UvrD (DNA helicase II) was overexpressed in *Escherichia coli* and purified as described previously (44). Purified recombinant hexahistidine-tagged BLM protein (45) was kindly provided by Dr. Ian Hickson (University of Oxford). T4 polynucleotide kinase was obtained from New England Biolabs.

Nucleotides, Oligonucleotides, and DNA Substrates—[^{3}H]ATP was from Amersham Biosciences, and [γ-^{32}P]ATP was from PerkinElmer Life Sciences. The oligonucleotides used for preparation of duplex DNA substrates are listed in Table I. Unadducted oligonucleotides were purchased from Lofstrand Technologies or Midland Certified Reagent Company. Oligonucleotide 25-mers containing diastereomerically pure *cis*- and *trans*-opened BcPh DE-dA adducts (Scheme I) were synthesized as their 5′-phosphates using a semi-automated procedure, essentially as described previously (Ref. 46 and references therein), with a manual step for coupling of the BcPh DE-dA-adducted phosphoramidites (47) as a mixture of their 1*R*/1*S* diastereomers. For details of the syntheses and chromatographic separation of the diastereomeric *R*/*S* pairs of *cis*- and *trans*-adducted oligonucleotides, see Supplemental Data. Absolute configurations were assigned to the separated (1*R* and 1*S*) oligonucleotide diastereomers by enzymatic hydrolysis (48) to the nucleoside adducts whose circular dichroism (CD) spectra were then compared with the known CD spectra (49) of the optically active BcPh DE-dA adducts. DNA duplex substrates were prepared as described previously (11) and are shown in Table II.

Helicase Assays—Helicase assay reaction mixtures (20 μl) contained 30 mM Hepes (pH 7.6), 5% glycerol, 40 mM KCl, 0.1 mg/ml bovine serum albumin, 8 mM $MgCl_2$, 2 mM ATP, 10 fmol of DNA duplex substrate (0.5 nM DNA substrate concentration), and the indicated concentrations of WRN, BLM, or UvrD. Helicase reactions were initiated by the addition of the respective helicase and then incubated at 37 °C for 15 min. Reactions were quenched with 10 μl of loading buffer (50 mM EDTA, 40% glycerol, 0.1% bromphenol blue, 0.1% xylene cyanol) containing a 10-fold excess of unlabeled oligonucleotide with the same sequence as the labeled strand. The products of the helicase reactions were resolved on nondenaturing 12% (19:1 acrylamide: bisacrylamide) polyacrylamide gels. Radiolabeled DNA species in polyacrylamide gels were visualized

TABLE I
Oligonucleotide sequences for DNA substrates

Benzo[*c*]phenanthrene DEs are covalently bonded to deoxyadenosine with the marker indicating the orientation of the intercalated hydrocarbon relative to the adducted base. For structures of the adducts, see Scheme I.

Oligonucleotide	Sequence	Adduct
A	5′—CCGGAACCGTTTAGCGGCCAAAGGC—3′	unadducted
B	5′—CCGGAACCGTTT◀ AGCGGCCAAAGGC—3′	*trans-R* BcPh DE-dA
C	5′—CCGGAACCGTTTA▶ GCGGCCAAAGGC—3′	*trans-S* BcPh DE-dA
D	5′—CCGGAACCGTTT◁ AGCGGCCAAAGGC—3′	*cis-R* BcPh DE-dA
E	5′—CCGGAACCGTTTA▷ GCGGCCAAAGGC—3′	*cis-S* BcPh DE-dA
F	5′—TTTTTTTTTTTTTTTTTTTTTTTTAATGGCC~ GCTAAACGGTTCCGG—3′	unadducted
G	5′—GCCTTTGGCCGCTAAACGGTATTTTTTT~ TTTTTTTTTTTTTTTT—3′	unadducted
H	5′—TTTTTTTTTTTTTTTTTTTTTTTTCCAAGTAAAA~ CGACGGCCAGTGC—3′	unadducted
I	5′—GCACTGGCCGTCGTTTTACGGTCGTGA~ CTGGGAAAACCCTGGCG—3′	unadducted

using a PhosphorImager and quantitated using the ImageQuant software (Molecular Dynamics). The percent helicase substrate unwound was calculated by the formula: percent unwinding = 100 × (P/(S + P)), where P is the product and S is the residual substrate. The values of P and S have been corrected after subtracting background values in controls containing no enzyme and heat-denatured substrate, respectively. Helicase data represent the mean of at least three independent experiments with mean ± S.D. shown by error bars.

For helicase sequestration studies, WRN (3.6 nM, 72 fmol) was preincubated with the indicated amounts (0–500 fmol) of "unlabeled" single-stranded DNA (oligonucleotides unadducted A or adducted E) or "unlabeled" forked-duplex DNA molecules (substrates 1-unadducted or 5-*cis-S*) in standard helicase reaction buffer (described above) containing 2 mM ATP for 3 min at 24 °C. 10 fmol of radiolabeled forked-duplex molecule (11-tracker) was subsequently added to the reaction mixture and incubated for 7 min at 37 °C. Reactions were then quenched and resolved on native polyacrylamide gels as described above. Typically, 75–90% of the tracker-11 helicase substrate was unwound in reactions lacking the competitor DNA molecule. Displacement (% Control) is expressed relative to the control reactions lacking the competitor DNA.

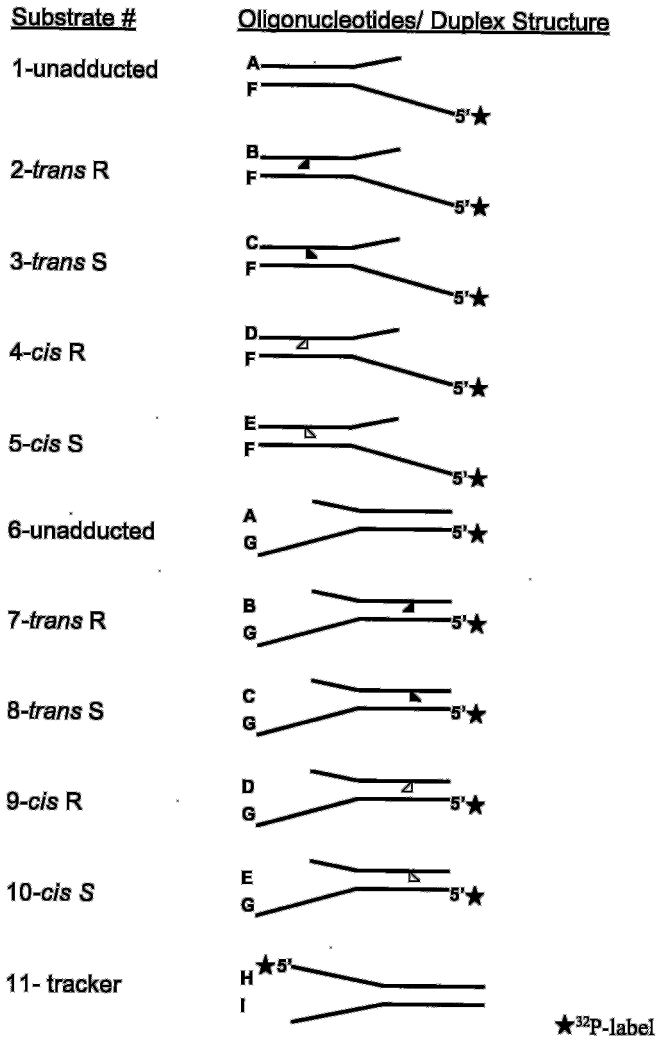
ATPase Assays—ATPase assay reaction mixtures (30 μl) contained 30 mM Hepes (pH 7.6), 5% glycerol, 40 mM KCl, 0.1 mg/ml bovine serum albumin, 8 mM $MgCl_2$, the indicated duplex DNA effector concentration, 0.8 mM [^{3}H]ATP, and 55 nM WRN. Reactions were initiated by the addition of WRN and incubated at 37 °C. Samples (5 μl) were removed at 2-min intervals and evaluated by thin layer chromatography as described previously (50). <20% of the substrate ATP was consumed in the reaction over the entire time course (10 min) of the experiments. K_{eff} values (Table III) were determined using linear regression analysis from double reciprocal plots of ATP hydrolysis initial rate *versus* forked-duplex DNA effector concentration (Fig. 4).

RESULTS

To understand better the effects of DNA structural elements on WRN-unwinding activity, we investigated how the helicase is affected by a series of structurally related single covalent DNA adducts. Specifically, the helicase substrates are forked DNA duplexes with a covalently bonded site-specific BcPh DE adduct in one of the two DNA strands (Table I) and positioned centrally in the 20-mer duplex region (Table II). For the purposes of this study, the stereochemistry and position of the adduct within the DNA duplex were varied. In the first class of substrates (Table II, substrates 1–5 with a 5-nucleotide 3′-unduplexed tail), the adduct is positioned within the shorter 25-mer strand on which WRN is presumed to 3′–5′ translocate. The second class of substrates (Table II, substrates 6–10 with a 25-nucleotide 3′-unduplexed tail) contains the same adducts as the first class, but the adduct is on the 25-mer strand opposite to the 45-mer strand on which WRN translocates. Because the forked-duplex substrates used in this study contain a relatively short duplex tract (20 bp), we were able to study WRN helicase activity by measuring the release of the labeled intact 45-mer in the absence of an auxiliary factor such as replication protein A. Our choice of forked-duplex DNA substrates harboring a site-specific BcPh DE adduct enabled us to assess the effects of adduct stereochemistry, orientation, and strand occupation on WRN helicase activity.

Inhibition of WRN Helicase Activity by a Single BcPh DE-dA

TABLE II
DNA substrates



Adduct in the Strand on Which WRN Translocates—We first tested helicase substrates with the single BcPh DE-dA adduct positioned centrally in the duplex tract on the strand on which WRN translocates (substrates 1–5). For all of the five substrates tested, the percent duplex DNA substrate unwound depended on the concentration of WRN present in the reaction (Fig. 1). For both the adducted and unadducted forked-duplex (20 bp) substrates, WRN exonuclease activity at the blunt end of the DNA substrate was minimal (<2%) as evidenced by the appearance of an intact released oligonucleotide on native gels (Fig. 1*A*) and confirmed by analysis of products on urea-denaturing gels (data not shown). Similar DNA unwinding of substrate 1-unadducted by an exonuclease-defective mutant WRN protein (WRN-E84A) was also observed (data not shown). These results are consistent with a previous observation that displacement of short (16 and 22 bp) duplex tracts by WRN helicase activity is more rapid than digestion by the WRN exonuclease activity (51).

Significant inhibition of WRN helicase activity by all four BcPh DE adducts was detected at all of the WRN concentrations tested, and the extent of helicase inhibition depended on the stereochemistry of the specific adduct (Fig. 1). Notably, the *cis*-opened adducts inhibited WRN unwinding more effectively than the *trans*-adducts (Fig. 1*B*). This difference was statistically significant at WRN protein concentrations of 2.4 and 4.8 nM. At a WRN concentration of 2.4 nM (Fig. 1*B*), the 5-*cis-S* adduct profoundly inhibited helicase activity resulting in only 2% of the substrate unwound compared with 23% of the 1-unadducted substrate. In comparison, the 3-*trans-S* substrate was unwound to an extent of 8%. At 4.8 nM WRN, 47% of the 1-unadducted substrate was unwound, whereas 31 and 14% of the 2-*trans-R* and 4-*cis-R* substrates, respectively, were unwound. Similarly, WRN unwinding of 22 and 11% was observed for the 3-*trans-S* and 5-*cis-S*-adducted substrates, respectively. These results indicate that the orientation (*cis*/*trans*) of the hydroxyl group at C-2 relative to the adenine 6-amino group at C-1 of the adducts had a significant effect on WRN helicase activity.

Absolute configuration (*S*/*R*) of the adducts at C-1 determines to which side (3′ or 5′) of the alkylated dA that the hydrocarbon intercalates. For substrates 1–5, the adducts with *S* configuration, *i. e.* oriented toward the 3′ end of the modified strand, are oriented toward the approaching helicase, whereas adducts with *R* configuration are oriented in the opposite direction, away from the approaching helicase. The adducts with *S* configuration exerted slightly greater inhibition of WRN helicase activity compared to adducts with the *R* configuration at 2.4 and 4.8 nM WRN (Fig. 1*B*). However, the differences are not statistically significant, indicating that for adducts on the strand on which helicase translocates the effect of orientation relative to the DNA strand was unimportant, particularly when compared with the effect of relative stereochemistry of the base and hydroxyl group on the adducts.

Orientation Effects of BcPh DE Adducts on WRN Helicase Activity Are Strand-specific and Stereospecific—We next tested the same WRN concentrations used for substrates 1–5 on the helicase substrates 6–10 in which the BcPh DE adduct resided in the displaced strand opposite to the strand on which WRN translocates. As observed for substrates 1–5, the percent of duplex DNA substrate unwound was dependent on WRN helicase concentration for these five substrates (Fig. 2). For all five substrates of this class, the intact oligonucleotide was released with minimal degradation from the blunt end by WRN exonuclease as observed on native gels and confirmed on urea-denaturing gels (data not shown). The percent displacement of 6-unadducted by WRN was ~3.5-fold greater than 1-unadducted at 1.2 nM WRN concentration (Figs. 1*B* and 2). In a previous study (11), it was shown that WRN unwound a forked duplex with a longer 3′-single-stranded DNA tail more efficiently than a substrate with a shorter 3′-single-stranded DNA tail, consistent with the difference between helicase activity on 1-unadducted with a 5-nucleotide 3′ tail and 6-unadducted with a 25-nucleotide 3′ tail.

WRN helicase activity on DNA substrates 7-*trans-R*, 9-*cis-R*, and 10-*cis-S* was inhibited compared with the 6-unadducted control substrate at all of the WRN concentrations tested (Fig. 2). At 1.2 nM WRN, ~2.4-fold inhibition of WRN helicase activity on 7-*trans-R, 9-cis-R, and 10-cis-S* was detected compared with 6-unadducted (Fig. 2). At 2.4 nM WRN, helicase activity was reduced 1.7- or 2.7-fold for 7-*trans-R* and 9-*cis-R*, respectively. In contrast, WRN unwinding of 8-*trans-S* was not affected at any helicase concentration. Thus, the WRN helicase is inhibited by *cis*-BcPh DE adducts on the displaced strand, although less so than by the same adducts on the opposite strand along which WRN translocates. The enzyme shows little or no discrimination between the *cis-R* and *cis-S* diastereomers. In contrast, the unwinding activity is unaffected by the *trans-S* adduct on the displaced strand but inhibited by the *trans-R* adduct. In substrates 6–10, the *R* adduct is intercalated toward the 5′ end of the displaced strand and is thus oriented toward on the 3′ end of the strand on which the

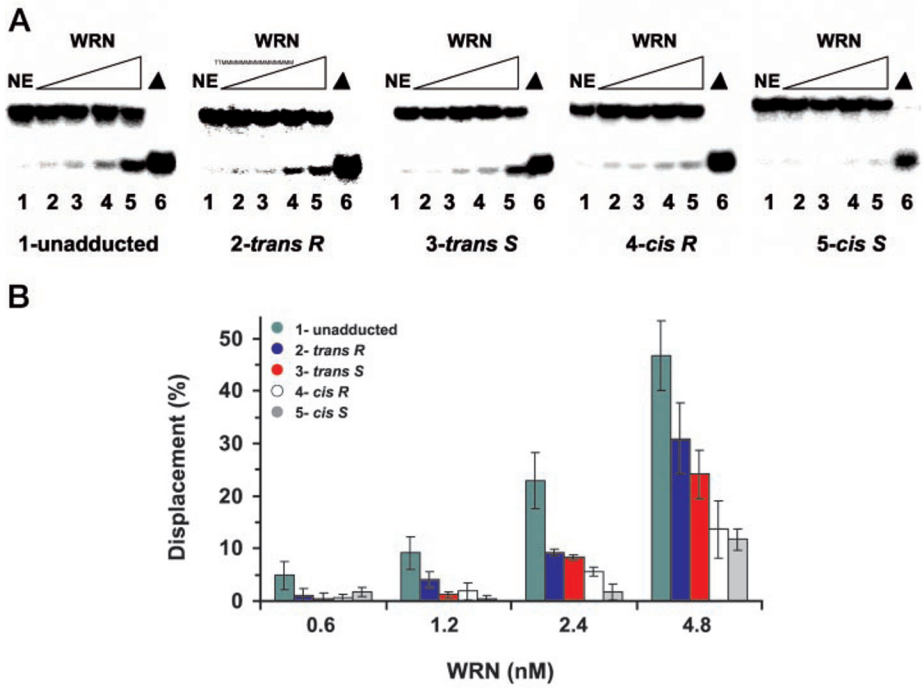


FIG. 1. **Inhibition of WRN helicase activity by a single BcPh DE adduct positioned in the strand on which WRN translocates is dependent on the stereochemistry of the adduct.** *Panel A*, reaction mixtures (20 μl) containing 10 fmol of the indicated forked-duplex DNA substrate and specified concentrations of WRN were incubated at 37 °C for 15 min under standard conditions. Products were resolved on native 12% polyacrylamide gels. Phosphorimages of typical gels are shown. For each gel: *lane 1*, no enzyme (*NE*); *lane 2*, 0.6 nM WRN; *lane 3*, 1.2 nM WRN; *lane 4*, 2.4 nM WRN; *lane 5*, 4.8 nM WRN; *lane 6*, heat-denatured substrate control. *Panel B*, percent displacement from *panel A* (mean value ± S.D. of at least three experiments) indicated by *error bars*.

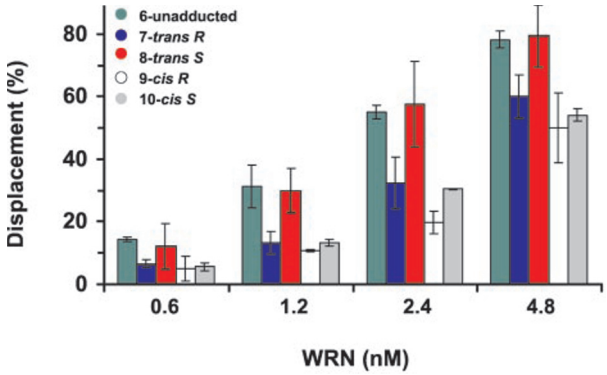


FIG. 2. **Inhibition of WRN helicase activity by a single BcPh adduct positioned in the strand that WRN displaces depends on stereochemistry and orientation of adduct.** Reaction mixtures (20 μl) containing 10 fmol of the indicated forked-duplex DNA substrate and specified concentrations of WRN were incubated at 37 °C for 15 min under standard conditions. Percent displacement (mean value ± S.D. of at least three experiments) indicated by *error bars*.

helicase is approaching (*cf.* Table II). The lack of inhibition of WRN helicase activity by the 8-*trans*-*S* substrate suggests that the *trans*-BcPh DE adduct residing in the strand opposite to the one on which WRN translocates only hinders WRN helicase activity when the adduct is oriented toward the advancing helicase.

WRN Is Not Trapped by BcPh DE-modified DNA—Previous studies have suggested that helicases are sequestered by DNA molecules containing various types of covalent lesions (for review, see Ref. 52). If sequestration by the present DE adducts in DNA occurs, preincubation of the WRN helicase with unlabeled single-stranded or forked-duplex DNA containing an adduct should trap WRN and prevent it from unwinding a labeled but unadducted forked-duplex tracker substrate. Based on the result that WRN is most significantly inhibited by the *cis*-*S* dA adduct residing in the strand on which the enzyme translocates, we chose this substrate to examine for potential WRN helicase sequestration.

When WRN was preincubated with either the adducted or unadducted single-stranded oligonucleotide, little to no inhibition of WRN helicase activity on the 11-tracker substrate was observed at any level of oligonucleotide tested (up to a 50-fold excess) (Fig. 3, *A* and *C*). In contrast, both the 1-unadducted and 5-*cis*-*S* forked-duplex DNA inhibited unwinding of the tracker duplex (Fig. 3, *B* and *D*). The ability of forked duplex but not single-stranded DNA to inhibit WRN helicase activity on a tracker substrate indicates that WRN preferentially recognizes or binds the forked structure. This result is consistent with the significantly increased helicase activity of WRN on a duplex substrate with 3′- and 5′-single-stranded DNA tails compared with a similar substrate flanked by only one single-stranded DNA tail (11). Preincubation of WRN with 100 fmol of the forked-duplex DNA molecules 1-unadducted and 5-*cis*-*S* resulted in a moderate decrease of WRN helicase activity. At higher levels of the 1-unadducted and 5-*cis*-*S* forked-duplex DNA molecules, WRN helicase activity on the 11-tracker substrate was inversely related to the amount of competitor DNA present in the preincubation step. At 200 fmol of either 1-unadducted or 5-*cis*-*S* competitor forked duplexes, DNA unwinding of the 11-tracker helicase substrate was approximately 55% of that observed in the absence of competitor duplex substrate, and at the highest level (500 fmol) of either 1-unadducted or 5-*cis*-*S*, unwinding of the tracker had decreased by ~80%. Notably, however, despite the observation (Fig. 1) that WRN helicase is significantly less active in unwinding the 5-*cis*-*S* forked duplex compared with the 1-unadducted substrate, preincubation with either of these two substrates resulted in an equal extent of inhibition of helicase activity on the 11-tracker (Fig. 3*E*). This result suggests that WRN was not preferentially sequestered by the forked-duplex molecule bearing the *cis*-*S* BcPh adduct relative to unadducted DNA.

WRN ATPase Activity in the Presence of Forked Duplex DNA Effectors—Because WRN ATP hydrolysis fuels DNA unwinding by the helicase, the inhibitory effect of the 5-*cis*-*S* BcPh adduct on WRN helicase activity raised the question of whether WRN ATPase activity might also be inhibited on binding of adducted DNA relative to unadducted DNA. To address this possibility, the initial rate of ATP hydrolysis was determined in the presence of the 1-unadducted and 5-cis-*S* substrates (Fig. 4 and Table III). For both the 1-unadducted and 5-*cis*-*S* forked duplexes, the dependencies of ATP hydrolysis rates on the concentrations of DNA effector from 10 to 200 nM were very

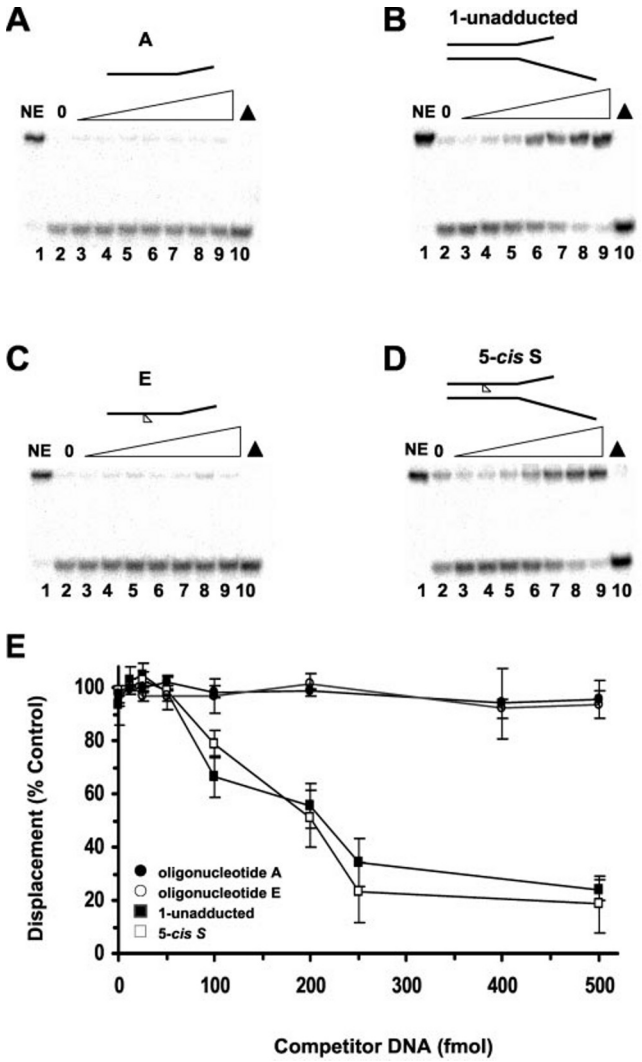


FIG. 3. **WRN is sequestered on BcPh DE-modified or unmodified forked-duplex DNA molecules.** Reaction mixtures (20 μl) containing 3.6 nM WRN (*lanes 2–8*) and increasing amounts of indicated oligonucleotides A (*panel A*) or E (*panel C*) (*lane 2*, 0 fmol; *lane 3*, 12.5 fmol; *lane 4*, 25 fmol; *lane 5*, 50 fmol; *lane 6*, 100 fmol; *lane 7*, 200 fmol; *lane 8*, 250 fmol; *lane 9*, 500 fmol) or increasing amounts of indicated forked duplexes 1-unadducted (*panel B*) or 5-*cis*-*S* (*panel D*) (*lane 2*, 0 fmol; *lane 3*, 12.5 fmol; *lane 4*, 25 fmol; *lane 5*, 50 fmol; *lane 6*, 100 fmol; *lane 7*, 200 fmol; *lane 8*, 400 fmol; *lane 9*, 500 fmol) were incubated at 37 °C for 3 min under standard conditions. After 3 min, 10 fmol of radiolabeled 11-tracker helicase substrate was added to each reaction and incubated for an additional 7 min at 37 °C. Helicase reaction products were resolved on native 12% polyacrylamide gels. *Lane 1*, no enzyme control (*NE*); *lane 10*, heat-denatured substrate control. *Panel E*, quantitative analysis of WRN helicase data is shown. ●, oligonucleotide A; ○, oligonucleotide E; ■, 1-undamaged; □, 5-*cis*-S. Data represent the mean value ± S.D. of at least three experiments indicated by *error bars*.

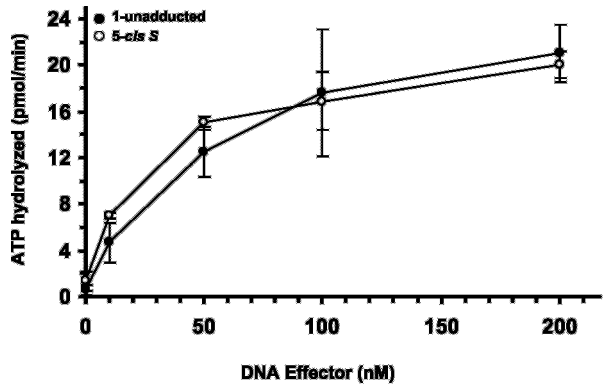


FIG. 4. **WRN requires similar concentrations of BcPh DE-modified and unmodified forked duplex for half-maximal ATP hydrolysis activity.** DNA-stimulated ATP hydrolysis reactions were as described under "Materials and Methods" using 55 nM WRN, 0.8 mM [^{3}H]ATP, and the indicated concentration of 1-unadducted (●) or 5-*cis*-*S* (○) forked duplex as the DNA effector.

TABLE III
Hydrolysis of ATP by WRN in presence of forked-duplex DNA effectors

ATP hydrolysis reactions were as described under "Materials and Methods" using 55 nM WRN and ATP at a concentration of 0.8 mM. For K_{eff} determinations, DNA effector concentrations ranged from 0 to 200 nM (forked-duplex molecules). For k_{cat} determinations, DNA effector concentration was 200 nM (forked-duplex molecules).

Name	*DNA Effector*	K_{eff} (nM) ± *SD*	k_{cat} (min^{-1}) ± *SD*
1-unadducted		25.2 ± 19	74.6 ± 6.7
5-*cis* S		21.2 ± 7.8	61.5 ± 9.3

similar (Fig. 4). The concentrations of forked-duplex DNA required to achieve the half-maximal rate of ATP hydrolysis (K_{eff}) for 1-unadducted and 5-*cis*-*S* substrates were 25 and 21 nM, respectively (Table III). The turnover rate constants (k_{cat}) for initial rates of ATP hydrolysis measured in the plateau region (200 nM forked-duplex DNA) were 75 and 62 min^{-1} for 1-unadducted and 5-*cis*-*S* BcPh DNA effectors, respectively. These k_{cat} values are in the same range as previously published values for WRN ATP hydrolysis using single-stranded DNA oligonucleotide effectors (43) under different reaction conditions. The similar K_{eff} and k_{cat} values for WRN ATP hydrolysis using BcPh DE-modified or unmodified forked-duplex molecules indicate that the presence of the BcPh DE adduct did not alter the allosteric effect of the bound DNA molecule on WRN ATP hydrolysis.

Effect of a BcPh Adduct on BLM and UvrD Helicase Activity—The inhibition of WRN helicase activity by the *cis*-*S* BcPh adduct positioned in the strand on which WRN translocates raised the question whether other 3′–5′-DNA helicases would be similarly affected or whether the inhibition was unique to WRN. Therefore, we tested BLM helicase, another human enzyme that is also a member of the RecQ family, and *E. coli* UvrD helicase, a member of Superfamily 1, on selected DNA substrates used for the WRN helicase characterization.

Two concentrations of BLM (2.6 and 5.2 nM), which were similar to the highest concentrations of WRN used, were examined. In the presence of 2.6 nM BLM, 19% 1-unadducted substrate was unwound by BLM, whereas only 8% 5-*cis*-*S* DNA substrate was unwound, a 2.4-fold reduction (Fig. 5*A*). At 5.2 nM BLM, 34% 1-unadducted substrate was unwound, whereas 15% 5-*cis*-*S* was unwound, a 2.3-fold reduction in BLM unwinding (Fig. 5*A*). Thus, BLM helicase activity was significantly inhibited by the presence of the *cis*-*S* BcPh adduct in the strand of the DNA substrate on which the helicase translocates. However, the inhibition of BLM helicase activity in the presence of this adduct was not as great as that observed (Fig. 1*B*) for WRN helicase activity (4.3–11.5-fold).

To address any strand-specific effects of the *cis*-*S* adduct on BLM helicase activity, we tested BLM on 6-unadducted, 9-*cis*-*R*, and 10-*cis*-*R* substrates. At 2.6 nM BLM, 25% 6-unadducted substrate was unwound compared with 28% 9-*cis*-*R* and 24% 10-*cis*-*S*, respectively (Fig. 5*B*). This lack of inhibition of BLM by these two substrates was clearly in contrast to the 2.7-fold reduction of WRN helicase activity by the 9-*cis*-*R* sub-

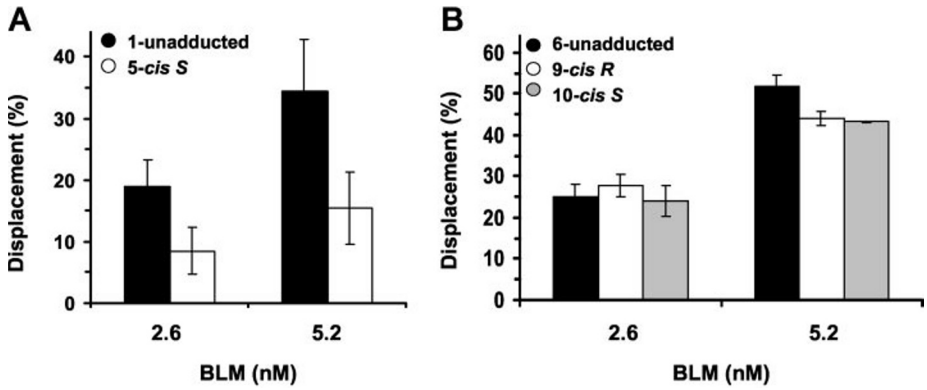


FIG. 5. **Effect of BcPh DE adducts on BLM helicase activity.** Reaction mixtures (20 μl) containing 10 fmol of the indicated forked-duplex DNA substrate and specified concentrations of BLM were incubated at 37 °C for 15 min under standard conditions. Quantitative analysis of BLM helicase data is shown. *Panel A*, 1-unadducted, *black column*; 5-*cis-S*, *white column*. *Panel B*, 1-unadducted, *black column*; 9-*cis-R*, *white column*; 10-*cis-S*, *gray column*. Data represent the mean value ± S.D. of at least three experiments indicated by *error bars*.

strate and the 2-fold reduction by the 10-*cis-S* substrate at the 2.4 nM WRN concentration. At 5.2 nM BLM, 53% 6-unadducted substrate was unwound compared with ~44% 9-*cis-R* and 10-*cis-S*. These results suggest that the unwinding activity of BLM helicase, in contrast to WRN, is inhibited only very weakly by a BcPh adduct positioned in the strand opposite to the one on which helicase translocates.

We next tested UvrD, a 3′–5′-helicase from Superfamily 1, to unwind the same helicase substrates (Fig. 6*A*). At 0.6 nM, UvrD unwound 16% 1-unadducted substrate compared with 9% of the adducted substrate 5-*cis-S*. At a 1.2 nM concentration of UvrD, the helicase unwound 27% 1-unadducted and 15% 5-*cis-S* substrates. The greatest inhibition of UvrD unwinding was observed at the highest UvrD concentration (2.4 nM). At this concentration, UvrD unwound 66% 1-unadducted substrate but only unwound 25% 5-*cis-S* substrate (Fig. 6*A*). Thus, similar to BLM helicase, UvrD was inhibited (~2.0–2.5-fold) by the *cis-S* BcPh adduct on the strand on which the helicase translocates although to a lesser extent than observed for WRN (4.3–11.5-fold, see above). UvrD was next tested on the helicase substrates, 6-unadducted as well as 9-*cis-R* and 10-*cis-S*, which have the adduct in the displaced strand. At 0.15 nM, UvrD unwound 30% 6-unadducted substrate compared with 18% 9-*cis-R* and 25% 10-*cis-S* (Fig. 6*B*). At 0.3 nM UvrD concentration, the helicase unwound 77% 6-unadducted, 50% 9-*cis-R*, and 62% 10-*cis-S* substrates (Fig. 6*B*). This inhibition by an adduct that resides in the strand opposite to the strand on which the helicase translocates is only slightly less than that observed with WRN (2–3-fold inhibition) and differs from our observation with BLM (≤20% inhibition).

DISCUSSION

We have examined the effects of bulky, well characterized, intercalating covalent adducts on WRN-catalyzed DNA unwinding. This is the first reported characterization of DNA unwinding by a RecQ helicase on duplex substrates harboring a site-specific covalent DNA modification. By using a set of duplex DNA substrates containing BcPh DE adducts at dA with defined stereochemistry, we have shown that WRN helicase activity is differentially affected by the relative and absolute configuration of the BcPh DE adduct residing in the double-stranded region of the forked-duplex DNA substrates.

WRN unwinding was clearly inhibited (up to ~11-fold) by a *cis-S* BcPh DE-dA adduct positioned centrally within the 20-bp duplex in the strand on which helicase translocates. Although not as dramatically, the same adduct positioned in the duplex region of the strand that WRN displaces exerted a significant 2.5-fold inhibition of helicase activity. Previous *in vitro* studies of the effects of helix-distorting lesions on the action of DNA helicases have largely suggested that a DNA lesion within a single strand inhibits DNA helicase activity when the lesion resides in the strand on which the protein translocates (52). A classic example of strand-specific inhibition was reported for Rad3, a 5′–3′-DNA helicase implicated in nucleotide excision repair and transcription. Rad3 helicase activity is sensitive to UV radiation damage in the DNA strand to which it binds and along which it translocates during the unwinding reaction but is not affected by UV radiation damage on the opposite strand (53). Strand-specific inhibition of Rad3 helicase activity was also observed for duplex DNA substrates containing either bulky cisplatin adducts that distort the helix or CC-1065-induced lesions that stabilize the helix (54). Strand-specific inhibition of unwinding activity by DNA lesions has also been observed for DNA helicases involved in DNA replication (for a review, see Ref. 52). The 1–2 intrastrand d(GpG) cross-link induced by the antitumor drug *cis*-diamminedichloroplatinum(II) significantly reduces the unwinding activity of the *Herpes simplex* virus type 1-replicative helicase UL9 only when it is present on the strand along which the protein translocates (55). The gene 4 protein that is essential for T7 viral replication translocates 5′–3′ along single-stranded DNA, and this movement is blocked by the bulky DNA adducts derived from benzo[*a*]pyrene (56). Benzo[*a*]pyrene DNA adducts only inhibit gene 4 helicase activity if they reside in the strand on which the helicase translocates (57). These *in vitro* studies suggest that for a number of helicases, a DNA lesion within a single strand compromises unwinding activity by preventing protein translocation on the strand containing the adduct.

The inhibition of WRN helicase activity by the BcPh DE adducts in the strand opposite to the one on which WRN translocates suggests that WRN is either sensitive to the adduct on the displaced strand or the shape and conformation of the DNA double helix encountered by the helicase during an unwinding reaction. In this mode, WRN might be sensitive to the aromatic rings of the intercalated phenanthrene residue and to the tetrahydrobenzo-ring projecting into the major groove or both as well as to more general distortions induced by the adducts in the DNA helix structure. We had previously investigated the effects of noncovalent DNA modifications on WRN helicase activity. Compared with the potent inhibition exerted by the minor groove binders distamycin and netropsin, WRN was not very sensitive to the DNA intercalators m-AMSA and actinomycin D and displayed only a mild sensitivity to the DNA intercalators ethidium bromide and mitoxantrone (58). NMR structural studies of mitoxantrone-DNA complexes suggest

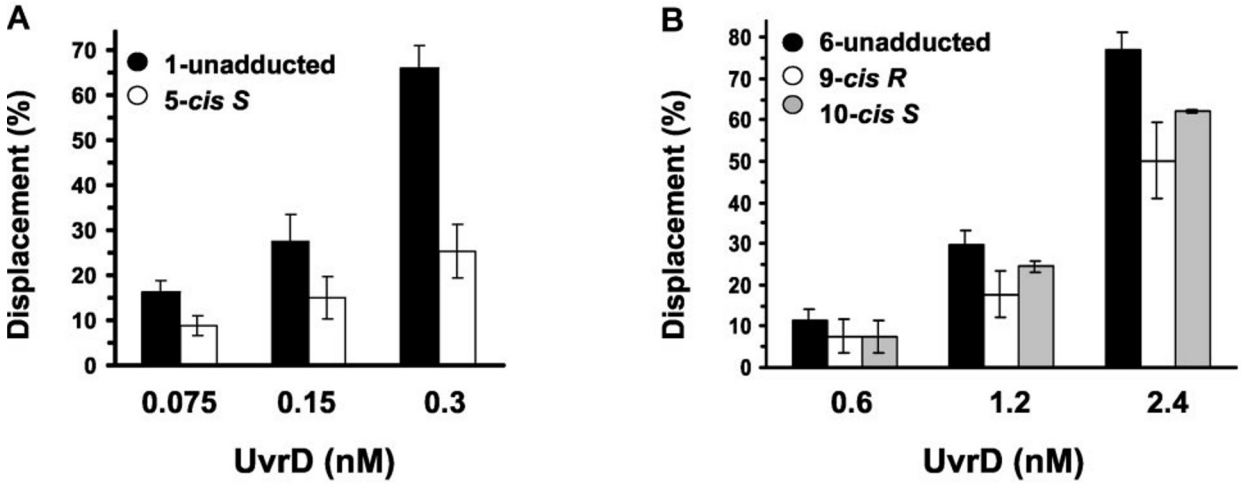


FIG. 6. **Effect of BcPh DE adducts on UvrD helicase activity.** Reaction mixtures (20 μl) containing 10 fmol of the indicated forked-duplex DNA substrate and specified concentrations of UvrD were incubated at 37 °C for 15 min under standard conditions. Quantitative analysis of UvrD helicase data is shown. *Panel A*, 1-unadducted, *black column*; 5-*cis*-*S*, *white column*. *Panel B*, 6-unadducted, *black column*; 9-*cis*-*R*, *white column*; 10-*cis*-*S*, *gray column*. Data represent the mean value ± S.D. of at least three experiments indicated by *error bars*.

that the compound intercalates between the base pairs as well as places functional groups (a positively charged amino group and two hydroxyl groups) in the major groove of B-form double helical DNA (59). Despite the chemical differences, the noncovalent interaction of mitoxantrone with B-form DNA shares some similarities with the covalent BcPh DE DNA adducts, which also intercalate between the base pairs and partially project into the major groove. However, the present covalent modifiers differ most significantly from non-covalent intercalators in that they cannot dissociate from the single-stranded portion of the DNA upon partial or complete unwinding. Thus, the sensitivity of WRN helicase to DNA structural perturbations may be influenced by the type of modification (covalent or noncovalent) as well as the nature of the perturbation of the DNA double helix.

The stereochemistry and orientation of the present covalent adducts also influence DNA unwinding by WRN helicase. For adducts positioned in the translocated strand, *cis*-opened BcPh DE adducts were significantly better inhibitors of WRN helicase activity than their *trans*-opened counterparts. Regarding orientation, we had hypothesized that WRN helicase activity might be inhibited to a greater extent by a BcPh adduct positioned in the direction of the advancing helicase. For the first group of substrates (1–5) where the adducts reside on the strand on which the enzyme translocates, the *S* adducts (3-*trans*-*S*, 5-*cis*-*S*) have the hydrocarbon intercalated toward the 3′ side of the modified adenine (in the direction of advance of the helicase), whereas the *R* adducts (2-*trans*-*R*, 4-*cis*-*R*) have the hydrocarbon intercalated in the opposite direction. As predicted, there appeared to be a slightly greater inhibition by 3-*trans*-*S* relative to 2-*trans*-*R* and by 5-*cis*-*S* relative to 4-*cis*-*R* (Fig. 1*B*). However, this effect of orientation was very small and in general was not statistically significant. A more significant difference was observed between the *trans* and *cis* adducts, such that *cis*-opened adducts were more inhibitory than *trans*-opened adducts. In the second group of substrates (6–10), which have adducts on the strand that is displaced, the hydrocarbon of the *R* adducts (7-*trans*-*R* and 9-*cis*-*R*) is intercalated toward the 5′ end of the modified strand and points toward the direction in which the helicase advances. In this series of substrates, the *trans*-BcPh adducts only inhibited DNA unwinding when directed toward the advancing helicase (7-*trans*-*R*). However, this effect of orientation was limited to the *trans* adducts. Thus, subtle changes in adduct stereochemistry or orientation can have a significant impact on DNA unwinding catalyzed by a helicase.

Despite the effective inhibition of WRN helicase activity by a single *cis*-*S* BcPh adduct in the strand along which the helicase translocates, the adducted forked-DNA duplex (5-*cis*-*S*) did not trap WRN to any greater extent than the unadducted duplex molecule (1-unadducted). Furthermore, DNA substrates containing the BcPh DE adducts are not degraded to any significant extent by WRN exonuclease, which can degrade from the blunt end of a forked duplex with a long (34 bp) duplex region (51), again suggesting that WRN protein does not become trapped on the damaged DNA molecule to provide an opportunity for WRN exonucleolytic digestion. These observations suggest that WRN dissociates as rapidly from the BcPh-modified DNA molecule as the unadducted DNA molecule. Other DNA lesions such as those induced by UV light, cisplatin, or diethyl sulfate (54, 60) have been shown to sequester Rad3, a helicase that functions during nucleotide excision repair. Similarly, the essential helicase for T7 replication, gene 4, when allowed to react with DNA containing a randomly introduced benzo[*a*]pyrene DE adduct on the strand on which the helicase translocates, is sequestered and forms a stable complex with the modified DNA (56, 57). The inability of a BcPh DE adduct in the strand on which the enzyme translocates to trap the WRN helicase suggests a possible difference in the mechanism of WRN inhibition by this adduct. Although BLM and UvrD helicase activities on the same adducted substrate were also reduced, the inhibition (2–3-fold) was not nearly as profound as that observed for WRN. This result suggests a difference between the ability of WRN and the other two 3′–5′ DNA helicases to unwind the BcPh-modified DNA that may translate to other types of lesions.

Despite the inhibition of unwinding, the presence of the BcPh DE adduct did not affect the rate of ATP hydrolysis relative to the unadducted control. Although it is possible that a helicase can retain ATPase activity even when sequestered by an inhibitory drug-DNA complex such as the anionic porphyrin *N*-methyl mesoporphyrin IX (61), in the present case preferential sequestration of the helicase by the adducted DNA was not observed (see above). The result showing that the kinetics of ATP hydrolysis by WRN with control and adducted DNA are essentially identical indicates that the *cis*-*R* BcPh adduct does not seriously perturb the allosteric interaction that activates the enzyme for ATP hydrolysis.

Our observations indicate that WRN helicase activity is significantly impaired by a single covalent DNA adduct on either strand although most strongly when the lesion is in the strand on which the enzyme translocates. This is in contrast to previous observations with T7 gene 4 helicase (56, 57) that was inhibited by benzo[*a*]pyrene DE-modified DNA only when the modification was on the translocated strand. Also in contrast to the T7 helicase with a benzo[*a*]pyrene DE adduct, the intact forked double-stranded DNA containing a BcPh DE adduct can readily dissociate from WRN. WRN then becomes available to bind another DNA substrate rather than stalling at the site of the lesion in a futile attempt to unwind it. It should be pointed out, however, that the present BcPh DE adducts were not a complete block to WRN since some unwinding of the BcPh-modified forked-duplex substrates occurred at higher WRN protein concentrations. The ability of WRN to unwind some but not all of the adducted duplexes may be accounted for by several factors/mechanisms. 1) In a subset of cases, WRN remains bound to the adducted DNA substrate long enough eventually to disrupt the duplex beyond the adduct. 2) Thermal breathing of the remaining intact duplex (10 bp) beyond the site of the BcPh lesion results in the slow non-enzymatic dissociation of these partial duplexes into single-stranded form. 3) Subsequent recruitment of additional WRN molecules at the site of a stalled helicase facilitates unwinding of the adducted DNA substrate. Although this study cannot distinguish among these possibilities, it is reasonable to conclude that despite the substantial decrease in WRN helicase activity in the presence of the BcPh DE adducts, the enzyme is not trapped by the adducted DNA substrate.

BcPh DEs react extensively with dA residues in DNA (37, 38), and the DEs and their resultant adducts induce a variety of mutations at dA sites *in vivo* (39, 63–65). These BcPh DE-dA adducts are of particular significance since they are known to elude cellular repair processes (66) and thus are highly likely to be encountered by other DNA-processing enzymes. The *cis-R* and *trans-S* dA adducts are derived from the highly carcinogenic and mutagenic (−)-(1*R*,2*S*,3*S*,4*R*)-DE enantiomer, whereas the *cis-S* and *trans-R* dA adducts are derived from the (+)-(1*S*,2*R*,3*R*,4*S*)-DE enantiomer, which is less carcinogenic (27, 40). Differences in the distribution of mutations induced by specific BcPh dA adducts have been observed (64), suggesting that the stereochemistry of these BcPh DEs may influence DNA-metabolic events such as the fidelity of translesion DNA synthesis (62). Although DNA polymerases are obvious targets, the ability of PAH-DE adducts in DNA to interact adversely with other enzymes such as topoisomerases (25, 26) may also contribute to their carcinogenic effects. Our present findings that helicases are inhibited by these adducts in a strand-specific and stereospecific manner suggest a further mechanism whereby these lesions could contribute to genetic damage and cell transformation.

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